The Impact of Storage Temperature on HCV RNA Detection by TaqMan Real-Time PCR

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The Impact of Storage Temperature on HCV RNA Detection by TaqMan Real-Time PCR

A thesis submitted in the partial fulfillment of the requirement for the degree of M. Phil in Biotechnology

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CERTIFICATE

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DEDICATION

"I dedicate this thesis to my parents and teachers, for teaching me with hard work and perseverance, almost anything is possible."

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My gratitude goes to ALLAH Almighty, the essence of my existence, all I have ever been and will ever be in life, I owe it all to you.

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LIST OF ABBREVIATION

Abbreviate	Extended form
%	Percentage
µg/µl	Micro gram per micro liter
µg/ml	Micro gram per milliliter
ALT	Alanine amino-transferase
bDNA	Branched-chain DNA
C	Core
CLIA	Chemiluminescence immunoassay
cDNA	Complementary DNA
CIAs	Chemiluminescence immunoassay
CMIA	Chemiluminescent microparticle immunoassay
DDAs	Direct acting antivirals
DS	Down-stream
ECLIA	Electrochemiluminescence immunoassay
EDTA	Ethylenediaminetetraacetic acid
EIAs	Enzyme immunoassays
ER	Endoplasmic reticulum
FDA	Food Drug Administration
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HVR	Hyper variable region
IRES	Internal ribosome entry site
IU/ml	International unit per milliliter
IVDU	Intravenous drug use
LOD	Limit of detection
MEIA	Micro-particle enzyme immunoassay
MHC	Major histocompatibility complex
Min	Minute
NAT	Nucleic acid test

NS	Non structural
NT	Not represented
°C	Degree celsius
ORF	Open reading frame
PCR	Polymerase chain reaction
RBV	ribavirin
RdRNAp	RNA Dependent RNA polymerase
RFLP	Restriction fragment length polymorphism
RIBA	Recombinant immunoblot assay
Rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase-PCR
SVR	Sustain virological response
TMA	Transcription mediated amplification
US	Up-stream
UTR	Untranslated region
WHO	World health organization

ABSTRACT

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family of viruses and is a main cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma. HCV genotype and viral load test are considered important prediction aspects determine interferon therapy response in chronic HCV patients. In routine analysis polymerase chain reaction (PCR) is the most commonly used test for HCV RNA amplification. Since for specimen screening, serum samples are frequently shipped to major cities, appropriate conditions are needed to preserve samples for the future reliability of HCV RNA testing. Therefore, proper storage and transportation conditions for the serum sample to detect HCV RNA are very important. Many authors have described that without proper condition it may cause false negative result. We have studied HCV RNA stability in serum samples taken from five hepatitis C positive patients under treatment. The samples were stored at different temperature before screening through real time PCR. (1) Immediate quantification, (2) storage at 4°C for 6h, (3) storage at 4°C for 12h, (4) storage at 4°C for 24h, (5) storage at 4°C for 48h, (6) storage at storage at room temperature (RT) for 6h, (7) storage at RT for 12h, (8) storage at RT for 24h, (9) storage at RT for 48h, (10) storage at 37°C for 6h, (11) storage at 37°C for 12h, (12) storage at 37°C for 24h, (13) storage at 37°C for 48h, (14) storage at 45°C for 6h, (15) storage at 45°C for 12h, (16) storage at 45°C for 24h, (17) storage at 45°C for 48h. HCV RNA was detectable in all the samples having different titre rate. A loss of almost 100% was observed in HCV RNA titre after 24 h and 48 h storage at 45°C. Insignificant loss in HCV RNA level was seen after 48 h storage at 4°C and 25°C. While significant decline of 1.36 $\log_{10}$ (31.4%) was observed after 48h storage at 37°C. The result from our study confirm that, serum sample can be shipped and store for 48h at 4 and 25°C without any significant decline while 37°C and more can cause significant decline in HCV RNA titre after 6 and 12h respectively.

Chapter 1

Introduction and Review of Literature

1.1 History of Hepatitis C virus (HCV)

HCV is a leading cause of liver disease infecting more than 185 million people worldwide (Hézode *et al.*, 2017). HCV was initially identified from an individual serum with non-A, non-B hepatitis by Choo et *al*. Shortly after HCV cloning, it was discovered to be the core reason of approximately 90% of non-A, non-B hepatitis in the US (Nouroz *et al.*, 2015). HCV is considered being the main cause of Liver disease further leading to liver cirrhosis and hepatocellular carcinoma(Wu *et al.*, 2013). Annually HCV effect about 3-4 million individuals (García-Samaniego *et al.*, 2001). Including 27% get cirrhosis and 25% have hepatocellular carcinoma (Nouroz *et al.*, 2015).

The reason behind hepatitis is virus however other events can also lead to hepatitis includes toxic and drug-induced, autoimmune, metabolic disorders, alcoholic and fatty liver (Svicher *et al.*, 2012). Presently six different variants of viral hepatitis are present, like hepatitis A, B, C D, E, and newly found Hepatitis G (Drane *et al.*, 2009). Hepatitis C causing death rates were 198,000 in 1990 which increase in 2013 to 358,000 (Abubakar *et al.*, 2015). Hepatitis C is thought to be the leading cause of parental transmitted, chronic and acute hepatitis and other hepatic disorders (Purcell, 1994). Hepatitis C has highly predominant to some parts of the world but the triggering agents is not completely understood. Generally the response of hepatitis C occurs after six to seven weeks however it may varies sometimes from 2 to 26 weeks depending on the quantity of viral genome in the blood and patient immune system (Seeff, 1991).

1.2 HCV genome and proteins

The HCV belongs to the genus *Hepacivirus* of the family *Flavivirdae*. HCV is an enveloped, spherical, positive sense single stranded RNA virus with diameter between 40 and 80nm in an infected patient (Goossens *et al.*, 2016). HCV included of almost 9600 nucleotides in length. In an HCV infected patient daily almost 10^{12} viral particles are generated (Kim and

Chang. 2013). The presence of extremely error prone RNA polymerase in HCV, it also shows significant genetic diversity and tendency for selection of immune evasion and drug resistance mutations (De Francesco and Migliaccio, 2005). The HCV genome contains one continuous open reading frame with non-translated regions (NTR) at 3′ and 5′ ends. The upstream coding region of HCV 5′ NTR contains 341 nucleotides and is composed of I to IV domains with extremely structured RNA elements comprising several stem loops and single pseudoknot (Brown *et al.*, 1992; Wang *et al.*, 1995). The internal ribosome entry site (IRES) is also present at 5′ NTR that induces the cap-independent translation of HCV RNA into single polyprotein (Honda *et al.*, 1996) by recruiting both cellular proteins and viral proteins such as eukaryotic initiation factors (EIF) 2 and 3 (Lukavsky *et al.*, 2000; Otto and Puglisi, 2004; Tang *et al.*, 2004). Depending on the genotype the HCV ORF contains 9024 to 9111 nucleotides. Single nucleotide encodes which further cleaved into 10 individual viral proteins by host and viral proteases (Alvisi *et al.*, 2011). HCV structure and its genome are shown in Figure: 1.1.

1.2.1 Structural proteins

The viral nucleocapsid protein is HCV core protein having several purposes involving RNA binding, cell signalling, immune modulation, autophagy and oncogenic potential. The core proteins also help in the making of HCV assembly by linking with the lipid droplets. The HCV E1 and E2 are enveloped glycoproteins present around the viral particles. Due to high degree of sequence variation, antibodies responses become ineffective when virus neutralizing antibody targeted HCV envelope, and contributes to HCV persistence (Kaplan *et al.*, 2007; Logvinoff *et al.*, 2004; Von Hahn *et al.*, 2007). The p7 protein is small ion channel present at the envelope region downstream which is essential for viral particle assembly and release (Von Hahn *et al.*, 2007).

1.2.2 Non-structural proteins

The key role in viral assembly played by NS2 viral autoprotease is, facilitating the cleavage among NS2 and NS3. The NS3 encodes the C-terminal RNA helicase-ntpase and N-terminal HCV serine protease (García-Sastre and Biron, 2006). For HCV antiviral drug

development, the NS3 region is the key target. NS4A with NS3 combine a stable complex and act as NS3 protease cofactor. The role of NS4B is not well assumed, however it is recognized for membranous web formation induction. NS5A act as dimeric zinc-binding metalloprotein which binds various host factors and viral factors closely to HCV core and lipid droplets. NS5A inhibitors of HCV showed antiviral effect in an infected individual and are in fast clinical development.

Lastly, NS5B is the RNA-dependent RNA polymerase (RDRP) the main target for antiviral drug development. Together these proteins also take part in various features of HCV life cycle, including HCV RNA translation, viral attachment, entry and fusion, HCV replication, posttranslational processing, virus assembly and release (Kim and Chang, 2013).

1.3 HCV life cycle

The life cycle of HCV is multidimensional and has not been totally understood. For the entry of HCV to the hepatocytes there are different types of molecules involved. Two enveloped glycoproteins (E1 and E2) and apolipoproteins exist at the surface of the lipoviroparticles with some other cell surface molecules. In initial low-affinity cell binding lycosaminoglycans and the LDL receptor likely to be involve. Then, E1–E2 binds with scavenger receptor class B member 1 and CD81, whereas occluding, claudin 1 and possibly other molecules such as claudin 9 or claudin 6, epidermal growth factor receptor or ephrin receptor type A2 are required for cell entry (Zeisel *et al.*, 2013). This multi-receptor complex facilitates uptake and describes organ and species specificity (Manns *et al.*, 2017). The part of immune dominant neutralization epitopes played by E2 envelope glycoprotein comprises hypervariable regions. Antibodies alongside these hypervariable regions are protective in patient sera. However, based on these viral proteins, successful prophylactic vaccine development is prevented due to high variability of HCV in same patient having different viral quasi-species. HCV receptor complexes are apparently linked with tight junctions, which assist direct cell–cell transmission (Manns *et al.*, 2017; Timpe and Mckeating, 2008). After attachment, entry of HCV into cells results in clathrin-mediated endocytosis, fusion between endosomal and viral membranes are followed, which further leads to the ejection of the nucleocapsid into the cytoplasm. The E1 envelope glycoprotein is supposed to be the fusogen

(the glycoprotein that facilitates cell fusion); (Manns *et al.*, 2017). The viral nucleic acid uncoat and releases the positive-strand genomic RNA into the cytosol, where it serves for synthesis of the HCV polyprotein as mRNA. The untranslated region of HCV 5′ contains an internal ribosome entry site, which controls translation HCV open reading frame (Honda *et al.*, 1999; Manns *et al.*, 2017). The long chain precursor polyprotein is generated which is translated at the endoplasmic reticulum membrane where further processing events take place, resulting in the generation of seven non-structural proteins and three structural proteins (Niepmann, 2013).

Two host cellular peptidases (signal peptide peptidase and signalase) are required for HCV structural proteins processing, while two viral peptidases (NS2 and NS3/4A) are involved for the processing of non-structural proteins. The HCV proteins remain connected through intracellular membranes after processing (Moradpour and Penin, 2013). The NS5B protein catalysed the replication. The helicase-ntpase domain of NS3 and the NS5A protein play an important regulatory part in HCV replication(Lohmann, 2013; Scheel and Rice, 2013). In RNA binding with a basic channel, NS5A acts as a dimer with a basic channel involved in RNA binding. NS5A protein's domain I and domain II are mandatory for HCV replication in the replication complex (Manns *et al.*, 2017). The NS3 helicase has significant role in separating the emerging and template RNA strands, displacing RNA-binding proteins and RNA secondary structures unwinding (Kapoor *et al.*, 2013). NS4B is a fundamental membrane protein having role in membrane rearrangements which is induced by membrane proteins, leading to the construction of the replication complex or 'membranous web' that compartmentalizes and support HCV replication. The genomic RNA positive strand act as a template for a negative-strand synthesis, then that negative RNA strand serve as template for production of numerous positive strands that are afterward helps for the translation of polyprotein, the synthesis of novel products of replication and packaging into fresh viral particles (Seigel *et al.*, 2013). Several host factors have also been involve in the HCV life cycle. The conformational changes that are mandatory for efficient HCV replication are inducing by the Cyclophilin A (also known as peptidylprolyl isomerase A) which binds to both NS5A and NS5B. An abundant liver-specific mirna-122 (mir-122), binds to two conserved sites of internal ribosome entry site, which is prerequisite for RNA stabilization

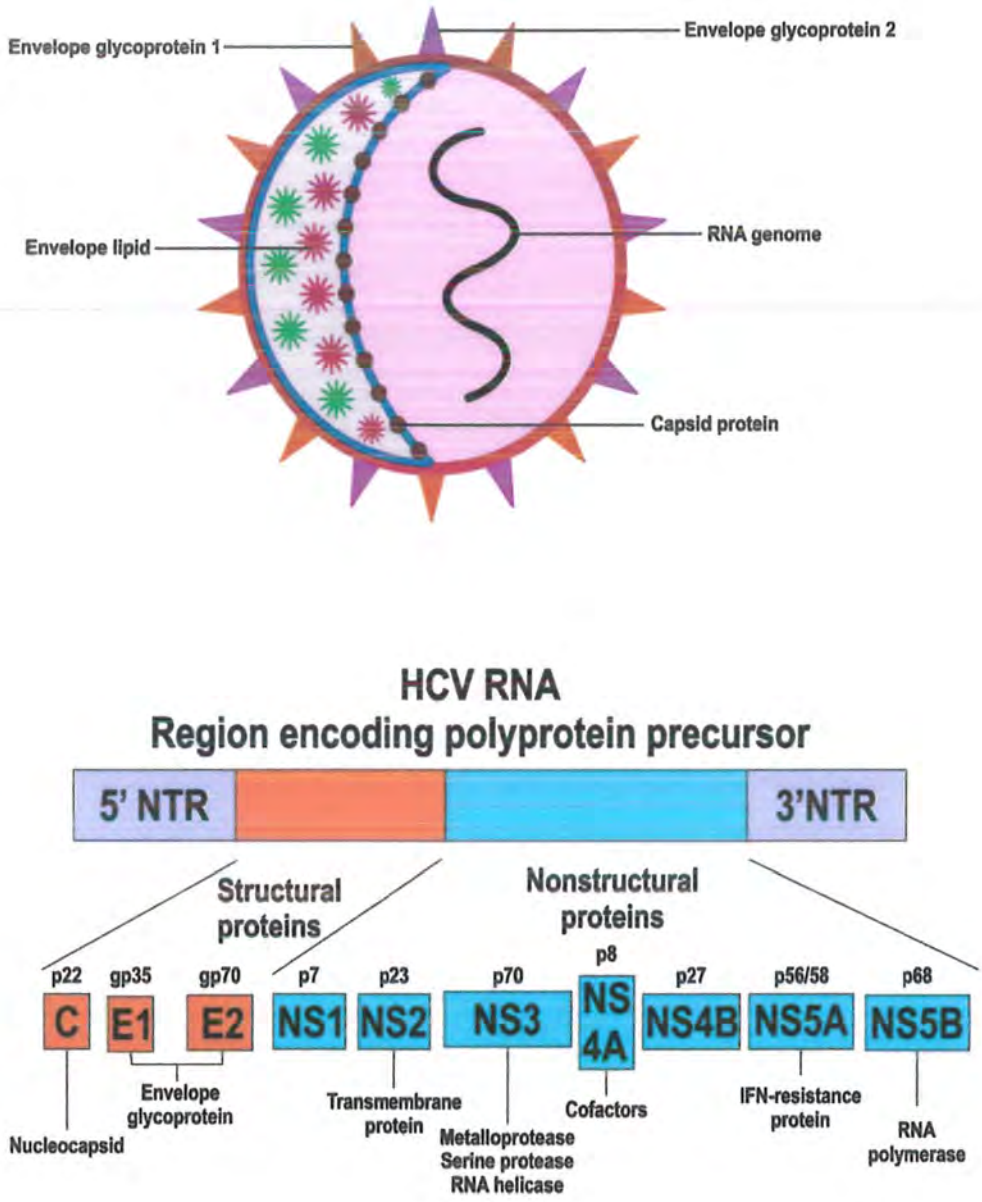

Figure: 1.1 HCV structure and its genome

The figure was reconstructed using CorelDraw X7 and adopted with slight modifications from (Knipe and Howley, 2013).

and efficient HCV replication. The initiation of viral particle formation starts from binding genomic RNA in cytoplasmic lipid droplets with the core and NS5A proteins. For assembly and release at the later stages the HCV uses the VLDL production pathway (Lindenbach and Rice, 2013; Manns *et al.*, 2017).

1.4 Global epidemiology of HCV infection

1.4.1 Prevalence estimates

Hepatitis C is a common infection worldwide; however, there is no data available to quantify the precise global burden of HCV due to poor surveillance system HCV (Thrift *et al.*, 2016). To estimate the worldwide prevalence of HCV, a study published in 2013 by the Global Burden of Diseases, Injuries and Risk Factors 2010 (GBD2010) from all the available literature. The study estimated that HCV infected >184 million people (tested through anti-HCV antibody), estimated>2.8% of the world population (Mohd Hanafiah *et al.*, 2013).These data are possibly underestimated as they ignore high-risk groups such as prisoners. Certainly, another study found that globally about 2.2 million HCV-infected individuals are in prison or in another closed institutions (Larney *et al.*, 2013). HCV is affecting more than 3.5% of each region population including Middle East, Central Asia, East Asia and North Africa as they are highly prevalent region of the world. Southeast Asia, Southern Latin and Central and America, Andean, Caribbean, Europe, Australasia, Oceania, and sub-Saharan African regions with moderate prevalence rate (1.5–3.5%), While seroprevalence of HCV is less than 1% in various Western European countries (UK, France, Denmark, Sweden, Germany and Switzerland). Asia–Pacific, Tropical North America and Latin America are the lowest prevalent regions with the rate of HCV <1.5%. Migrants to Europe and North America from Asia, Eastern Europe and sub-Saharan Africa make up an enormous number of HCV cases (Greenaway *et al.*, 2015). Highest rate of HCV infected people are living in Asia, almost >100 million individuals with >50 million people each from south and East Asia, Including >15 million from North Africa and Middle East, 10 million live in Western Europe and >11 million people in Southeast Asia. Assumed that 20–40% of individuals with acute HCV patients clear their infection spontaneously, probably 20-40% of the individuals are having

active viremia showing lower seroprevalence through different region (Bruggmann *et al.*, 2014; Saraswat *et al.*, 2015).

1.4.2 Age and gender distribution of HCV

The patterns of prevalence in all region of the world across age groups are similar with few exceptions on the basis of 2005 data. In common, prevalence increases with growing age until a highest prevalence at 55-64 years in maximum regions. However, there is an additional earlier peak in the prevalence curve at age 15-19 in western sub-Saharan Africa, Likewise, in Australasia, the first peak of prevalence in ages 20-24 years and then in ages 55-64 years. Most of the countries, men are having highest prevalence rate than women due to highest number of intravenous drug users. However, in France, women are highly infected than men (Bruggmann *et al.*, 2014). Likewise in Germany, women with age >69 years are highly prevalent than men with same age. The risk factors in both the countries, during childbirth women were infected via contaminated blood or equipment in the late 1970s. In Turkey, the HCV infection are more common in women then man, more women are hospitalized than men showing nosocomial infection(Bruggmann *et al.*, 2014).

1.4.3 Prevalence of HCV genotypes

There are six HCV genotypes available with 30-35% nucleotides differences in their genome, further leading to subtypes in each genotype. Each genotype have specific treatment regimens, cure rates and duration of treatment including clinical course (Kanwal *et al.*, 2014). Therefore, worldwide treatment policy depends upon the understanding of genotypes prevalence in that specific region. The most prevalent is HCV genotype 1 globally, covers 83.4 million of all (46.2% of all HCV patient). In USA and Europe the most common is HCV subtypes 1a and 1b, whereas in Japan, about 73% of HCV patients are having 1b.The next most prevalent is genotype 3 with 54.3 million cases (30.1%) globally. There are more consequences related to high prevalence of HCV genotype 3 compare to other genotypes, because HCV genotype 3 causes high risk of mortality and other serious hepatic complications genotypes(Kanwal *et al.*, 2014). Genotypes 2, 4 and 6 cause 22.8% of HCV cases, while genotype 5 contributes <1%. Variation found in HCV genotypes in different

parts of the globe. However, a few geographical arrangements can be distinguished (Messina *et al.*, 2015). HCV genotype 1 is the major type in 85 of 117 countries, HCV genotype 3 is commonly found in South Asia and comparatively rare in Africa, frequency of genotype 4 is the highest which present from Middle East to Central Africa, genotype 5 is specifically prevalent only in southern Africa, genotype 6 is also comparatively limited in its geographical extent, with highly prevalent in East and Southeast Asia, and the only prevailing genotype in Laos. The HCV genotype diversity varies across different regions and countries. Diversity is high in china and many other Southeast Asian countries, Australia and the Western Europe suggesting that infections are more evenly dispersed through several genotypes 3 and 6 (Thrift, 2016).

1.5 HCV transmission and prevention

Before the documentation of HCV, the main route for transmission of HCV was blood transfusion or blood-related products. In USA and several European countries, the risk of HCV transmission almost eliminated from blood transmission due to screening of routine Anti-HCV antibodies. However, there is great variation in routine testing globally for transfusion-related infections. In ~40 countries testing is not conducted at all with unreliable testing system in many other countries (Lemoine and Thursz, 2014). In low-income and middle-income countries RNA-based testing is extremely uncommon for active infection. In some of the high prevalent countries (such as Pakistan, Egypt and Eastern Europe) the key source of infection is nosocomial infection (Chlabicz *et al.*, 2004; Kandeel *et al.*, 2012; Safi *et al.*, 2012). WHO estimated that each year unsafe injections causing ~2 million new infection (Hauri *et al.*, 2004). Large-scale investment in empowerment of the general public, Education and awareness campaigns about consequences of reusing the medical instruments is the key to keep people safe from this huge problem. Another critical step for each country is to keep testing and identifying at-risk person for HCV. However, the scariest part about HCV infection, that 90% of the individuals infected by HCV do not know about their disease (Hatzakis *et al.*, 2011; Osburn *et al.*, 2010). Currently, there are no proper recommendations available from the WHO for population-based screening for HCV, however, WHO recommended screening for high risk group where HCV infected population, are well defined. Like, in the USA, Screening is recommended for USA birth group so called baby boomers

who were born between 1945-1965, which revealed 73% of HCV infection prevalence (Smith *et al.*, 2012). With the availability of extremely efficacious treatments, improvement and rapid implementation programmes that overcome the many hurdles to diagnosis, screening, linkage to care and treatment for HCV are immediately needed(Norton *et al.*, 2016; Re *et al.*, 2014).

1.6 HCV treatment

Although uncommon, but interferon-based HCV treatment is successful which slow down the progression of liver disease and significantly reduce however, not eradicate the risk of cirrhosis and HCC (Arase *et al.*, 2013; Trinchet *et al.*, 2015; Van Der Meer *et al.*, 2012). Direct acting antivirals (DAAs) have potentially enhanced the treatment response in comparison with pegylated interferon-α and ribavirin(Hofmann and Zeuzem, 2011). First generation NS3 and NS4A protease inhibitors like Telaprevir and Boceprevir have been increase the rate of Sustained Virological response (SVR) above 70% (Zeuzem *et al.*, 2011). Drug resistance and extensive side effects are the key factors that influence the effective treatment(Sullivan *et al.*, 2013). Simeprevir and Paritaprevir the NS3-NS4A inhibitors, Daclatasvir and Ledipasvir the NS5A inhibitors and Sofosbuvir the NS5B polymerase inhibitor are currently used DAAs, also known as Viekira Pak. According to specific genotype the DAAs effect differently (Sarrazin *et al.*, 2015). The success rates are attained up to 90% due to highly genetic variability toward antiviral resistance (Nelson *et al.*, 2015). Costly and re-infection occurrence after spontaneous clearance needs advance and affordable DAAs drugs (De Vos and Kretzschmar, 2014). The genetic diversity causing drug resistance during treatment or baseline are not well defined so far. The breakthrough in HCV research since the discovery of the disease is presented in the (Figure 1.2).

1.7 Serological test for HCV

Hepatitis C cannot be controlled without proper screening programme nationally. WHO highly recommended the screening for people who receive blood or blood products and also people having history of intravenous (IV) drug use (Razavi *et al.*, 2014). HCV infection has been diagnosis through two types of assays. Indirectly diagnosis, that detect HCV specific

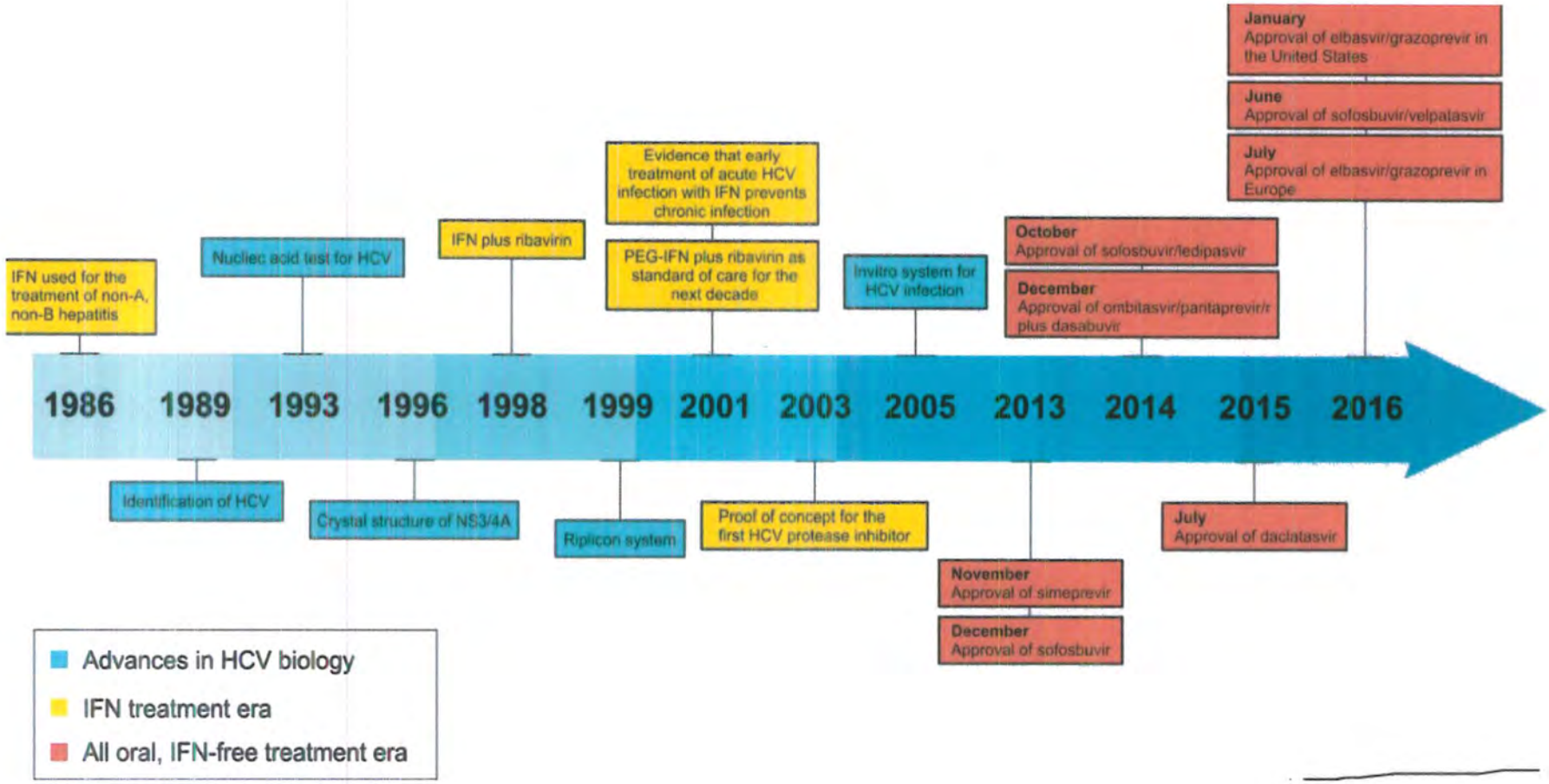

Figure: 1.2 Milestones in HCV research and management.

The figure was reconstructed using CorelDraw X7 and adopted with slight modifications from (Knipe and Howley, 2013).

antibodies and direct detection that identify viral components {*e.g.* the viral genome or core antigen, (Saludes *et al.*, 2014)}

1.7.1 Indirect diagnosis

Serologic assays are used to diagnose and screen HCV exposure and detect HCV-specific antibodies (IgM and IgG). However, these assays are unable to differentiate among active and resolved infections.

A. Screening assays

From 1989, after the discovery of HCV and identification of their immunodominant epitopes, HCV infection has diagnosed from serum sample via detecting HCV antibodies using enzyme immunoassays (EIA). Later on, the developments of serological assays make possible third-generation assays to use HCV recombinant antigens from the core regions, NS3, NS4 and NS5 (Alborino *et al.*, 2011). Currently, fully automated immunoassays with random access instruments and high-throughput are extensively using in clinical diagnostics laboratories (Saludes *et al.*, 2014). Features are summarized in Table 1.1 of the most commonly used assays.

B. Confirmatory assays

To check the existence of HCV specific antibodies, using Recombinant immunoblot assays (RIBA) for already positive EIA tested patient, specifically when screening low prevalent populations HCV infection. On a single band on a membrane strip, RIBA measure the existence of several HCV proteins which shows their specificity. This assay includes recombinant proteins and synthetic peptides from the E2 hyper variable region, the NS3 (helicase), NS4A, NS4B and NS5A regions (Saludes *et al.*, 2014).

C. Rapid point-of-care screening tests

Point-of-care test (POCTs) is the easiest and quickest method to detect HCV infection (Branson, 2007). For HCV specific antibodies detection several pocts have been developed comparatively highly specific and sensitive (Shivkumar *et al.*, 2012). The only PDA approved

test is oraquick HCV Rapid assay (orasure). In this test the NS3, NS4 and core antigens are immobilized on nitrocellulose membrane, and in the presence of HCV specific antibodies after 20 min the results are directly visualized which generate a reddish purple line, using colloidal gold labelled with protein A. This test showed specificity of 99.5%-99.6% and sensitivity of 97.8%-99.3% (Smith *et al.*, 2011). Another hepatitis C test system Home Access (Home Access Health Corporation) declare by the FBA, which shows high specificity and sensitivity (Saludes *et al.*, 2014).

1.7.2 Direct diagnosis

For active HCV detection and quantification there is need to detect viral components.

A. HCV core antigen screening and quantification

In the serum of infected patients the core region of HCV can be detected, and its quantity are related HCV-RNA (Bouvier-Alias *et al.*, 2002; Descamps *et al.*, 2012; Park *et al.*, 2010). Compare to molecular assays the HCV Core assays costs less and easily performed in an immunoassay format. But we cannot quantify the exact number of HCV genome in this kind of test (Mederacke *et al.*, 2009; Morota *et al.*, 2009). Several reports have recommended that the core region quantification might be used to diagnose the response to IFN-α plus RBV therapy in chronic HCV patients (Buti *et al.*, 2004; Rendón *et al.*, 2005; Vermehren and Sarrazin, 2012).

1.8 Molecular HCV assays

Molecular HCV assay is used for the detection of HCV genome in an infected patient as well as use for other purposes in the clinical settings. Molecular assays are having three different stages in case of HCV diagnosis(Liver, 2011). First, it is required for an early acute HCV infection diagnosis, before the detection of specific antibodies (within 1-3 week after exposure), the HCV RNA can be detected. Second, molecular assays are used for the detection of HCV RNA in HCV infected patient (lower limit of detection is < 50IU/ml), having already positive for antibodies test. Finally, the chronic stage of HCV is confirm by

Table: 1.1 Main commercial immunoassays to detect anti-hepatitis C virus antibodies approved for in vitro diagnostics

Analyzer and manufacturer	Assay principle	HCV antigen	Solid phase	Time of reaction (min)	Reaction sample volume (µL)	IVD registration
LiaisonXL, DiaSorin	CLIA	Core, NS3, NS4	Paramagnetic particles	46	25	CE
AxSYM, Abbott Laboratories	MEIA	HCr43 (Core), c200 (NS3), c100-3 (NS4A)	Paramagnetic particles	30	33	FDA, CE
Elecsys, Roche Diagnostics	ECLIA	Core, NS3, NS4	Paramagnetic particles	18	40	FDA, CE
VITROS ECi, VITROS 3600, Ortho-Clinical Diagnostics	CLIA	c22-3 (Core), c200 (NS3 and NS4), NS5	Microwell	55	20	FDA, CE
ADVIA Centaur, Siemens	CLIA	c22-3 (Core), NS3, c200, NS5	Magnetic particles	58	10	FDA, CE

IVD: Certified as in vitro diagnostic test or device; FDA: Food and Drug Administration (United States of America); CE: ConformitéEuropéenne European Union); CMIA: Chemiluminescent microparticle immunoassay;ECLIA: Electrochemiluminescence immunoassay; HCV: Hepatitis C virus;CLIA: Chemiluminescence immunoassay; MEIA: Microparticle capture enzyme immunoassay (Saludes V et al., HCV).

Table: 1.2 Hepatitis C virus-RNA qualitative assays approved for in vitro diagnostics

Assay and manufacturer	Method	Lower limit of detection (IU/mL)	Reaction sample volume (µL)	Instrumentation for automated processing	IVD registration
COBAS® AmpliPrep/COBAS® TaqMan® HCV Qualitative Test v2.0, Roche Molecular Systems	Real-time RT-PCR	15	650	Fully automated: cobas p 630 Instrument (primary tube handling), COBAS® AmpliPrep (extraction and MM setup).	CE, FDA
COBAS® AmpliPrep/COBAS® AMPLICOR HCV Test v2.0, Roche Molecular Systems	RT-PCR	50 (plasma) 60 (serum)	250	COBAS® AmpliPrep (extraction), COBAS® AMPLICOR® Analyzer (amplification and detection)	CE, FDA, Canada
APTIMA HCV RNA Qualitative Assay1, Hologic - Gen-Probe	TMA	5.3	500	Not automated. PANTHER System's functionality currently in development	FDA
COBAS® AMPLICOR HCV Test v2.0, Roche Molecular Systems	RT-PCR	50 (plasma) 60 (serum)	500	COBAS® AMPLICOR® Analyzer (amplification and detection)	CE, FDA, Japan,
VERSANT® HCV RNA Qualitative Assay, Siemens	TMA	5.3	50	TMA modules (TCS, luminometer HC+, *etc.*)	CE, FDA

The performance of this assay has not been demonstrated for monitoring hepatitis C virus (HCV) infected patients. IVD: Certified as *in vitro* diagnostic testor device; FDA: Food and Drug Administration (United States of America);CE: ConformitéEuropéenne (European Union); MM: Master mix; RT-PCR: Reverse transcriptionpolymerase chain reaction;(Saludes *et al.*, 2014) .

the presence of both the HCV RNA over six months and HCV antibodies {(with the exception of highly immunosuppressed patients) (Chevaliez, 2011; Ghany *et al.*, 2011; Orlent *et al.*, 2012; Saludes *et al.*, 2014)}. Approved commercially available assays for *in vitro* diagnostics are listed in Table 1.2.

1.8.1 HCV genotyping assays

Genotyping of HCV is mandatory for tailor dose and treatment duration as well to predict the response of IFN-α-based therapy (Ghany *et al.*, 2009; Saludes *et al.*, 2014). Currently for genotyping, sequence analysis of specific regions (NS5, 5-UTR, E1 and core) is considered to be the most authentic(Athar *et al.*, 2015; Ohno and Lau, 1996). However, alternate methods that offer cost-effective and fast genotyping are more appropriate for clinical use. These amplification methods include type-specific probes or type-specific primers(Athar *et al.*, 2015; Okamoto *et al.*, 1992; Qu *et al.*, 1994), line-probe testing (Stuyver *et al.*, 1996), restriction fragment length polymorphism (Lau *et al.*, 1998), and heteroduplexmobility analysis (Athar *et al.*, 2015; Margraf *et al.*, 2004; White *et al.*, 2000).

1.9 Stability of HCV RNA at different temperatures during handling and storage

In addition to the regularly performed nucleic acid amplification techniques (NAT)-based screening may decrease the titre of HCV in specimen due to different storage and transportation conditions. Several reports studied (Alter, 1997; Busch *et al.*, 1992; Cardoso *et al.*, 1999; Cuypers *et al.*, 1992; Halfon *et al.*, 1996; Han *et al.*, 1991; Kuo *et al.*, 1989; Miskovsky *et al.*, 1996; Quan *et al.*, 1993)the HCV RNA stability in samples from diverse origin (blood, serum and plasma) in the presence of many preservatives (EDTA, citrate etc.) at various storage temperatures (-20, -70, 4 and room temperature). These studies used different analytical methods for covering several handling condition. Considering all these factors sometimes they reach apparently different results which not quit surprised. However, Several many improvements have been attained during last years concerning NAT standardization, comprising comparability of results and control stability between different laboratories (José *et al.*, 2005).

The HCV RNA given the best preservation result when stored in whole blood sample for five weeks through TaqMan real-time PCR (Grant *et al.*, 2000; Sener *et al.*, 2010).Damen

et al studied the effect of whole blood when put in EDTA and PPT after 72 hours and adding mix coagulants (CPDA,EDTA), however no significant decline observed. Another study conducted by (Damen *et al.*, 1998)reported no decline rate after 14 days in HCV RNA titre.

The best preservation results in previous studies suggested at room temperature (25°C) were, for 120 hours when anticoagulants EDTA or CPDA-1 added to whole blood (Grant *et al.*, 1999). Different studies reported different results after storage at 25°C like Cuypers reported that rapid 3 to 4 log units decline rate was observed in HCV titre in whole blood and serum within 14 days when stored at RT (Cuypers et al, 1992) while Jose *et al* reported that no change in HCV titre occur after 14 days in plasma samples the HCV titre didn't change (Jose *et al.*, 2003).Minor loss of 0.2 $\log_{10}$ occurred after 72 hours when stored at 25°C in PPT and EDTA tubes (Grant et al., 1999) while 100 percent loss showed when kept at RT for 5 days (Halfon et al., 1996) and in frozen serum the HCV RNA decreased 20% after 4 days of storage (Krajden*et al.*, 1999). Kamili*et al* reported that the HCV survival rate was up to 16 h (Kamili*et al.*, 2007).

HCV RNA stability did not disturb when sample store at 37°C for up to 12h before refrigeration (Grant *et al.*, 1999) and after one day of storage at 37°C HCV RNA start degradation (Krajden*et al.*, 1999). There is significant decline observed of 0.37 $\log_{10}$ when anticoagulants CPDAA-1 and EDTA mixed with whole blood after 120h (Grant *etal.*1999) while Krajden*et al* reported more than 20% loss after 4 days (Krajden*et al.*, 1999).

1.10 Effect of sample storage conditions on virus titre in Pakistan; Background of this study

Present study was planned to check the stability of HCV RNA titre at different storage temperatures in order to investigate the effect of transportation and environmental conditions. In Pakistan, major diagnostic labs are in the bigger cities, where samples are transported from sub-urban and rural areas. From our collaboration with different diagnostic labs in Islamabad, we learnt that the travelling time for some samples varied depending upon area of collection; such as 5 to 10 hrs for the samples received from Mardan, Peshawar, Jehlum, DI Khan, Lahore, Buner, Attock, Faisalabad, Multan etc. However, some samples took up to 48 hrs (Chatral, Dir, Bajawar, Zhob, and some parts of Afghanistan like Kandahar, Kunduz etc.) of travelling times. Most of the samples reached to labs as serum form collected as blood in serum separator tubes. The weather of Pakistan fluctuates in different seasons of the year,

from 0°C in winter up to 48°C in summer (Naqvi and Rehmat, 2009). The source of sample reaching to the lab is non-refrigerated vehicles based; in which temperature directly affect the objects. That is the reason behind our study to include different temperatures like 4°C, 25°C, 37°C and 45°C.

1.11 Aims and objectives of study

i. Collection of HCV positive serum samples and quantification of viral titre

ii. Optimization of real-time PCR protocol using standard samples

iii. Exposure of selected samples with known virus titre to a range of temperature

iv. Quantification of viral titre from each sample after heat exposure

v. To check the stability of HCV RNA after temperature exposure by comparison with control samples suing real time real-time PCR

vi. Application of statistical tools in order to interpret results in terms of temperature effect

Chapter 2

Materials and Methods

Table: 2.1 List of enzymes and chemicals

Material	Manufacturer
Primer	Sansure Biotech
Probe	Sansure Biotech
dNTPs	Sansure Biotech
Mg^{2+}	Sansure Biotech
Taq Polymerase	Sansure Biotech
ROX Solution	Sansure Biotech
PCR buffer	Sansure Biotech
Manganese acetate	Sansure Biotech
Viral RNA isolation Kit	MACHEREY-NAGEL
Ethanol	Merck

Table: 2.2 List of equipment

Equipment	Manufacturer
Pipettes	
P10	Eppendorf
P100	Eppendorf
P100	Eppendorf
Eppendorf Tips	Biosafe Technologies
Centrifuge	Thermo
Vortex Machine	Scilogex
Incubator	Memmert

2.1 Collection of samples and exposure to multiple temperature conditions

Blood samples were collected from 5 HCV infected patients with informed consent in the serum separator tubes (Fisher Scientific SST) and then spin at 3000rpm for 10min to collect the serum. The sera were distributed into aliquots and were stored at these temperature conditions: (1) immediate quantification, (2) stored at 4°C for 6h, (3) stored at 4°C for 12h, (4) stored at 4 °C for 24h, (5) stored at 4°C for 48h, (6) stored at room temperature (RT) for 6h, (7) stored at RT for 12h, (8) stored at RT for 24h, (9) stored at RT for 48h, (10) stored at 37°C for 6h, (11) stored at 37°C for 12h, (12) stored at 37°C for 24h, (13) stored at 37°C for 48h, (14) stored at 45°C for 6h, (15) stored at 45°C for 12h, (16) stored at 45°C for 24h, (17) stored at 45°C for 48h.

2.2 Extraction of RNA from sera

RNA extraction was performed by Viral Nucleic acid isolation Kit (GenePro) with slight modifications. For extraction of HCV RNA first 150µl serum was added to the nuclease-free 1.5mL micro centrifuge tubes of each sample, with the addition of 600µl of freshly prepared lysis Buffer (carrier RNA-supplemented Binding Buffer). For better results these were mixed for 15sec by pulse-vortexing immediately. The next step was the incubation of samples for 5min at 72°C followed by addition of 600µl of ethanol (100%). Vertex the samples for few seconds followed by centrifugation to remove drops from inside of the caps. The fluid from microcentrifuge tube was transfer to the spin column tube and centrifuged for 1min at 8000rpm. Filter tube was separated from collection tube after centrifugation and the collection tube containing the flow through was thrown away. The spin column tube was put in to another collection tube used for the remaining sample and centrifuged it at 8000rpm for 1min. Then the column was put in to new collection tube, and 1st wash RAW buffer 500µl was added in the upper reservoir and centrifuge for 1mint at 8000 × g. In second wash, Buffer RAV3 of 600µl was poured to the spin column tube and centrifuged it for 1min at 8000rpm.

For third wash, again Buffer RAV3 of 200µl was added to the spin column and centrifuged at 11000rpm for 5min. Spin column containing specimen were put into a clean 1.5mLcentrifuge tube and 50µl of RNase-free water was added (70°C pre-heated). Incubated for 1min at room temperature following centrifugation at 11000rpm for 1min and stored for further use at -20°C. For rest of the sample same protocols were applied to extract total HCV RNA.

2.3 Real-time PCR mediated HCV RNA quantification

The Mini Opticon (BIO RAD Real-Time PCR system, USA) was used as mentioned in the manufacturer's instructions for quantification of viral loads. In short, after HCV RNA extraction each specimen was transferred to BIO RAD Mini Opticon for realtime amplification.

2.3.1 PCR amplification of 5' UTR

In the HCV genome 5' UTR is highly conserved, therefore this region can be used to determine HCV RNA in each sample.

2.3.2 Reaction mixture

For HCV 5′ UTR specific primers were available in the HCV Mix. First the 5′ UTR was reverse transcribed into cDNA and then the cDNA was amplified. Total Reaction Volume was 30µl, including 25µl of Master mix and 5µl of viral RNA (Sansure Biotech).

2.3.3 PCR program

Real-Time PCR (Mini Opticon, Bio rad, USA) was used for amplification and detection of viral RNA. PCR program was customized as, pre-heated was performed for 1min at 95°C. Then cDNA formation occurs at 50°C for 30min. Then after cDNA formation step, 45 repeating cycles of denaturation, annealing and extension were performed. Denaturation of the cDNA was performed at 95°C for 15sec, annealing and extension temperatures were kept 60°C for 30sec.

2.4 Statistical analysis

Statistical operations with the data were performed using IBM SPSS Statistics version available online.

Table: 3.1 Variation in HCV RNA in serum sample after several storage conditions measured

HCV RNA viral load (log)																
	4°C				25°C				37°C				45°C			
0 hr	6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h	6 h	12 h	24 h	48 h
5.28	5.27	5.27	5.24	5.20	5.21	5.13	5.10	5.13	5.08	4.41	4.26	4.37	3.30	3.27	2.86	2.55
5.18	5.17	5.17	5.16	5.12	5.16	5.04	5.10	5.13	4.77	4.24	3.43	3.29	4.06	3.49	2.14	1.09
4.85	4.85	4.84	4.82	4.83	4.83	4.62	4.77	4.41	4.24	3.79	2.81	2.62	3.90	3.31	3.29	2.58
4.81	4.80	4.78	4.77	4.63	4.79	4.77	4.68	4.52	4.71	4.48	4.35	3.77	3.76	3.64	3.18	2.19
4.32	4.32	4.28	4.26	4.23	4.27	4.21	4.03	4.01	3.79	3.75	2.85	2.71	3.19	2.22	1.81	1.74

At time point 0, the HCV RNA mean basal concentration was 4.88log (from 4.32 to 5.28log). After 6h of storage at +4°C, the circulating HCV RNA mean concentration was 4.882log (from 4.32 to 5.27log). After 12, 24 and 48h at 4°C the mean concentration of circulating HCV RNA were respectively 4.868, 4.850 and 4.802log (from 4.23 to 5.27log). No decline rates were observed in any period at 4°C with a mean change of 0.032log from time point 0 to 48 hrs. These data are shown in Table and Figure 3.1.

HCV RNA levels were analysed after storing at room temperature but no significant decline was observed in any of the samples. The aliquots were stored for time period of 6, 12, 24 and 48h and their results were 4.852, 4.754, 4.640logs, respectively (Figure 3.2). The mean change observed after 48h were 0.212logs which is not significant.

3.2 Stability of HCV RNA in positive serum samples stored at 37°C

Five HCV serum sample solution were exposed to nucleic acid extraction following real-time PCR assay respectively when all the samples were collected. The result of quantitative PCR indicated that HCV RNA started to degrade within this time period at 37°C, respectively (Figure 3.3). Same protocols were applied to these samples by storing at 37°C for 6, 12, 24 and 48h.

At time point 0, the mean concentration of HCV RNA was 4.888log. After 6h of storage at 37°C, the mean concentration of circulating HCV RNA was 4.518log (from 3.79 to 5.08log), showing the decrease of $0.37\log_{10}$. The mean value of HCV RNA titre after 12h of storage conditions were 4.134log with a change of $0.75\log_{10}$. Similarly, after 24 and 48h, the mean values of HCV RNA were 3.540 and 3.525log, respectively. This shows the difference of 1.34 and 1.36log after PCR quantifications.

3.3 Stability of HCV RNA in positive serum samples stored at 45°C

Twenty dilutions of starting material from five samples were followed-up for 6 to 48h at 45°C. The HCV RNA content was quantified at different storage time periods by real-time PCR. HCV RNA titres decreasing significantly after each time periods. Decrease in HCV RNA values of each sample at 37°C was more than 1log in the samples. After 6 hrs at 45°C the HCV RNA titre altered from the mean value $4.888\log_{10}$ to $3.642\log_{10}$, difference up to $1.246\log_{10}$ is highly significant.

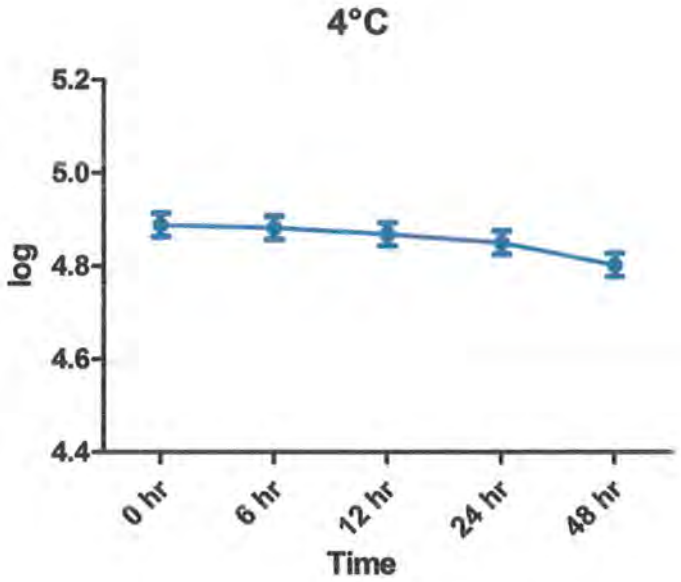

Figure: 3.1 Changes in HCV RNA levels in serum specimens subjected to 4°C

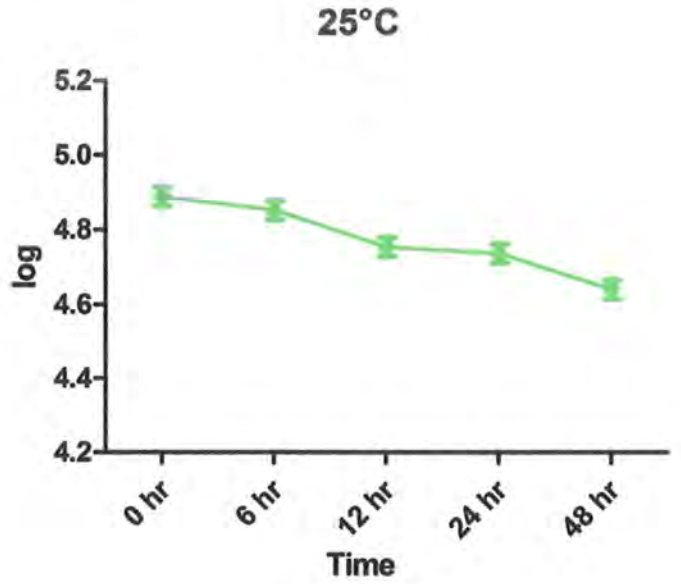

Figure: 3.2 Changes in HCV RNA levels in serum specimens subjected to 25°C

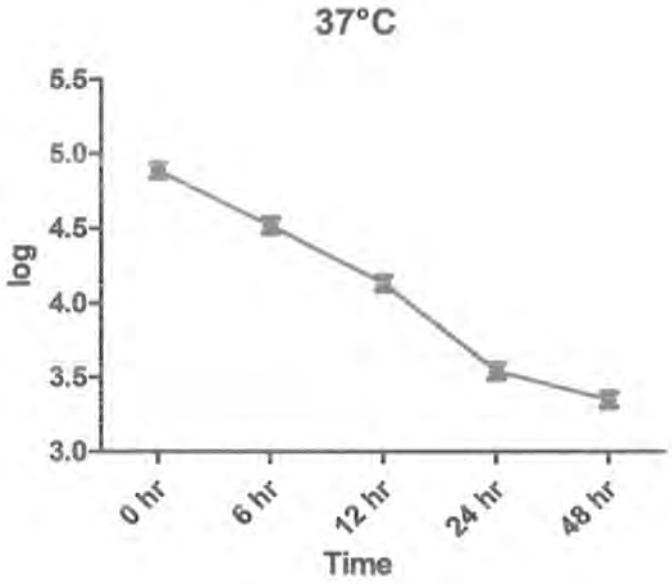

Figure: 3.3 Changes in HCV RNA levels in serum specimens subjected to 37°C

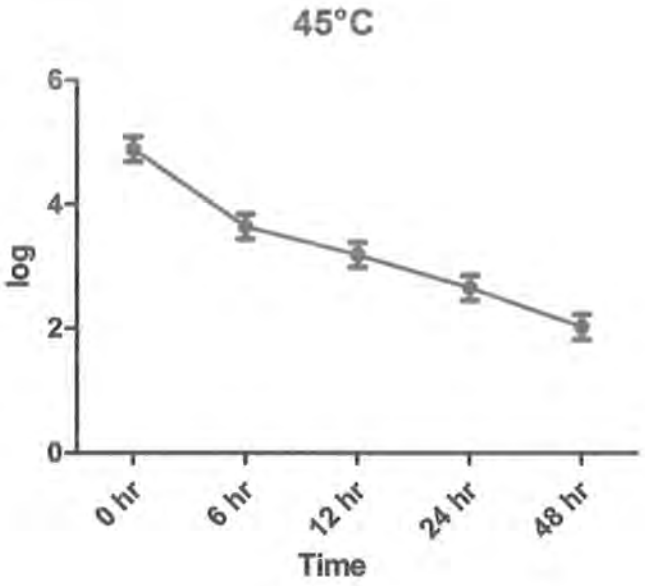

Figure: 3.4 Changes in HCV RNA levels in serum specimens subjected to 45°C

The result after 12h of storage was 3.186 log_{10}, showing 1.702log difference. After 24 and 48 h the mean value of HCV RNA titre were 2.656 and 2.030, respectively. The change observed after 24h of storage was 2.232log, and 2.858log after 48h, which is quiet enormous (Figure 3.4).

The overall changes in HCV RNA titre after 6, 12, 24 and 48h of time period of storing HCV serum samples at 4, 25, 37 and 45°C is shown below (Figure 3.5)

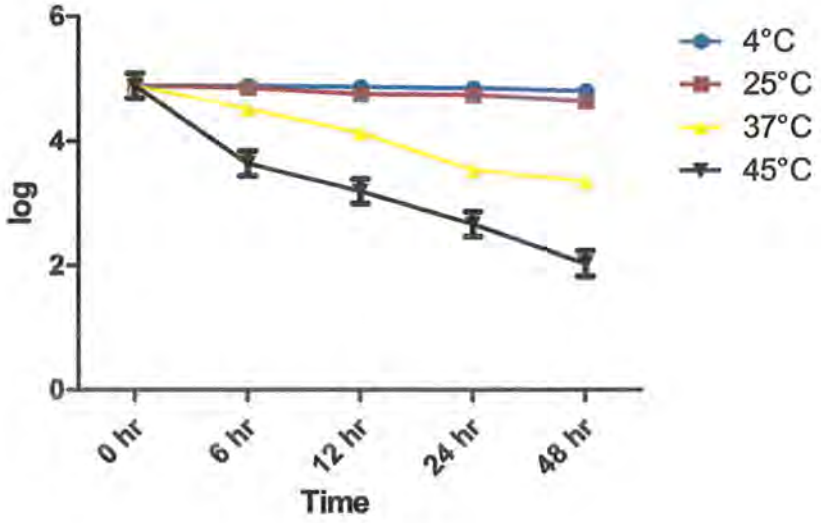

Figure: 3.5 Changes in HCV RNA levels in serum specimens subjected to different temperature condition.

Chapter 4

Discussion

HCV RNA detection in blood is a key diagnostic and screening test. Additionally, for the response to anti-HCV therapy HCV RNA is considered as the main predictor. So many studies reported inappropriate storage temperatures leads to change viral load measurement and decrease HCV RNA stability. Several reports are available on the effect of storage and different handling conditions on HCV RNA stability in blood, plasma or serum samples.

The fluctuation in temperature in these studies are from very low temperature (-20, -80 etc.) up to 37°C or they also studied aliquots of same sample expose to different temperatures with different dilutions (Cuypers HTM et al, 1992; Halfon P et al, 1996; Quan CM et al, 1992; da Silva Cardoso M et al, 1999; Wang JT et al, 1992; Grant PR et al, 2000).Using various methods of quantification (COBAS AmpliPrep/COBAS TaqMan, TaqMan Real Time PCR, Branched-DNA Technology etc.) have been reported as well (Jose M et al, 2003; Gessoni G et al, 2000; Damen M et al, 1998; de Gerbehaye AIM et al, 2002; Krajden M et al, 1999). The effect of clots and anticoagulants on HCV PCR has extensively been studied (Cuypers H et al, 1992; Manzin A et al, 1994; Damen M et al, 1998).

To our Knowledge, there is no such study conducted which reports the stability of HCV RNA titre at 45°C. In our study, we used real-time PCR for HCV RNA viral load quantification. Five HCV positive patients' samples were taken, which includes three male and two female patients. They were basically taking interferon based treatment for HCV and were on the middle of the treatment. After taking the samples we divided it into 17 aliquots and store at different temperature conditions for different time periods while the control sample were put at -20°C until the time of analysis. Four of the aliquots were thawed at 4°C and were left respectively after 6, 12, 24 and 48h and then put it back at -20°C for further analysis. Same protocol followed for the rest of the aliquots at 25, 37 and 45°C. For measurement of HCV RNA quantitative test BIO RAD Mini Opticon were used.

Several reports are available to explain the effect of storage on stability of HCV RNA at different environmental conditions (i.e. -80°C, -70°C, -20°C, RT, 4°C, 37°C, FT) from diverse origin (e.g., serum, plasma and blood) (A. Gerbehaye et al., 2002; LEE ET AL., 2002; Sener et al., 2010; M-A. Trabaudet al., 1996; M. Damen et al., 1998; M. Jose´ et al.,

2005; S. Kamili et al., 2007; M. Jose et all., 2003; Wang et al.,1992; M. Krajden et al., 1999; J. Greenman et al., 2015; Comert et al., 2013;) and also reported when added different preservatives (Perez et al., 2010; Lee et al., 2002).

For HCV RNA testing mostly the samples are taken in EDTA plasma or in Serum form. However, sometimes different anticoagulants (i.e., heparin, sodium citrate etc.) are added to increase the stability of HCV RNA, but on the other side these anticoagulants have inhibitory effect on the PCR, and in this case heparin is considering mostly used anticoagulants (Damen et al., 1998; wang et al, 1992).

Some literature show controversial results comparison to other reported studies. Some reports showed that HCV RNA is stable at 4°C for 4 days. However, with increasing temperature the concentration become decrease (Krajden M et al, 1999). One report suggested that the HCV RNA is stable in serum samples are stable for 3 days at both 4°C and room temperatures (Trabaud MA et al, 1996; F. Comert et al. 2013). Another report showed that after three to eight FT cycles the HCV RNA stay stable in serum samples (Davis GL et al, 1994; Krajden M et al, 1999).

There are different arguments regarding the stability of HCV RNA at RT. Some studies reported that storage at room temperature, the HCV RNA is unstable and showing 100% loss after five days (Cuypers HT et al, 1992; Busch MP et al, 1992; Halfon P et al, 1996).While other study shows no decline in HCV RNA after storing at room temperature ((Quan et al., 1992; Grant et al. (2000); Fong, 1993; Wang et al., 1992; Trabaud*et al.*, 1996). These differences were linked with sample handling, number of samples and the amount of viral load (Krajden et al., 1999a).

Various studies are available to show the HCV RNA stability at 4°C storage. One study reported the stability at 4°C stored in clotted blood for 72h (Damen M et al, 1998). Another study showed long term storage at 4°C after different dilution for 21 days, the HCV RNA was still detectable by using qualitative test (Da Silva Cardoso et al, 1999). Da Silva Cardoso suggested that the sample types (serum, blood, plasma) have small effect on the concentration of HCV RNA after storage for 168 days at 4°C (Da Silva Cardoso et al, 1998). Further research done by Gessoni et al, stated that after 5FT cycle and 168h of storage in plasma sample the result showed in insignificant decline rate (Gessoni et al, 2000).

We studied the response of viral load after different time periods (6h, 12h, 24h and 48h) at 4°C. With time point 0, the recovery of HCV RNA was 99% at any point without any significant decrease. In our study, compare to time point 0 no significant decline were observed after storing at 25°C for 48h ($p<0.05$). The results were checked after different time period (6h, 12h, 24h and 48h) with respect to starting viral load at room temperatures and noted the variation respectively (0.03, 0.13, 0.15 and 0.24 $\log_{10}$). After two days the difference were noted as 0.24 log, compare to another study by Comert et al reported that after 72h at 25°C the difference was observed 0.2 $\log_{10}$ (F. Comert et al., 2013). The study showed insignificant decline rate when storing at room temperature for up to 48h storage.

In molecular assays the variation of less than 0.5$\log_{10}$ IU/mL are usually accepted to be not taken into account (Sebire et al., 1998; Pawlotsky, 1997). Kessler *et al* determined that HCV RNA are remain stable in serum for 96h at RT when stored in NASTs (nucleic acid stabilizer containing tubes) (Kessler HH et al, 2001). Different collection tubes were used in different studies with different processing techniques which makes there comparison difficult. Overall reports depicts that HCV RNA remain stable for 72h when store at room temperature in serum and in plasma samples, as they showed not much difference when store in each of them.

The result showed when store at 37°C for 48h while inspecting after different time periods (6hrs, 12hrs, 24hrs and 48 hrs). With respect to time point 0 the mean viral load of HCV RNA of 5 samples were decreased up to 31.4% in viral load of 1.53$\log_{10}$ after 48h. After short time periods (6, 12 and 24h) there was no big difference in the results, which showed 7.5%, 15.4%, 27.5% loss in the viral load. Previously reported studies based on 37°C storage condition in serum sample showed 105% loss of HCV RNA after 4 days (*M. Krajden et al 1999).* Grant et al reported insignificant decline of 0.25$\log_{10}$ when store HCV RNA at 37°C in whole blood cells after 120 h (P.R. Grant et al, 2000). The reason behind the study reported by Grant et al was the storage in whole blood cell.

In addition, there are no such reports available to discuss the HCV RNA stability in serum samples at 45°C. Typically, it's supposed that at 45°C temperature HCV RNA containing serum sample could be degraded.

In this current study planned to find out the stability of HCV RNA at different time periods by storage at 45°C. These samples show different HCV RNA titre in respective time

period. After 6hrs of time period they showed 1.24$\log_{10}$ (25.49%) decline in RNA titre. Similarly after 12h of time period the titre decreases 1.70$\log_{10}$ (34.82%). Likewise after 24h and 48h time period the HCV RNA titre decline 2.23$\log_{10}$ and 2.85$\log_{10}$ (45.66% and 58.47%) Low titre of HCV RNA in serum is less stable compares to high level of RNA titre in the serum, therefore in our study the decline in log values are high compare to already reported studies (M. Damen et al, 1998).

The result of our study here reported that there is no significant effect on serum sample at 4°C and at room temperature for 48 hrs. However, it is necessary to keep the serum sample at a cold place during transportation and storage in summer specifically 37°C and above it. As it is causing highest decline rate in of HCV RNA titre in serum sample.

Chapter 5

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